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Human Embryonic Stem Cells: Culture, Differentiation, and Genetic Modification for Regenerative Medicine Applications

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ABSTRACT

Human embryonic stem (hES) cells can proliferate extensively in culture and can differentiate into representatives of all three embryonic germ layers in vitro and in vivo. The undifferentiated hES cells have now been cultured for more than 50 passages in vitro, yet maintain a normal karyotype. The hES cells express a series of specific surface antigens, as well as OCT-4 and human telomerase, proteins associated with a pluripotent and immortal phenotype. On differentiation, OCT-4 and human telomerase expression decreases with the emergence of a maturing population of cells. During hES cell differentiation, modulation of the expression of many genes has been evaluated using microarray analysis. To improve the ease, reproducibility, and scalability of hES culture, methods have been developed to propagate the cells in the absence of mouse embryonic cell feeders. hES cells maintained in culture using extracellular matrix factors together with mouse embryonic cell conditioned medium proliferate indefinitely while maintaining a normal karyotype, proliferation rate, and complement of undifferentiated cell markers. hES cells cultured without feeder layers retain their capacity to differentiate into cells of all three germ layers in vitro and in teratomas. The hES cells can also be genetically modified transiently or stably using both plasmid and viral gene transfer agents. These analyses and technological developments will aid in the realization of the full potential of hES cells for both research and therapeutic applications. *(Cancer J 2001;7(Suppl 2):S83–S94)*

KEY WORDS

Embryonic stem cell, differentiation, regenerative medicine, cell therapy

In 1998, Thomson et al[1] described the first isolation of human embryonic stem (hES) cells. The hES cells were derived from the inner cell mass of excess embryos produced for clinical in vitro fertilization procedures. The inner cell masses were cultured on mouse embryonic feeder cells and were established as cell lines, which have been propagated as undifferentiated cells in culture. Clonal cell lines have also been established.[2] After long-term culture, the hES cell lines have a normal karyotype and express several markers that are characteristic of human embryonal carcinoma and primate embryonic stem cells, namely SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Oct 4.[1] In addition, the undifferentiated hES cells constitutively express telomerase, an occurrence that is supportive of the fact that these cells can be propagated in culture indefinitely.[1]

hES are pluripotent, retaining the ability to differentiate into cells that represent all three germ lineages of the body.[2] In addition, hES-derived differentiated cells retain their ability to form complex organized tissues. When the hES cells are injected into immunocompromised mice, teratomas are observed with evidence of organized endodermal, mesodermal, and ectodermal primitive tissues.[1] Moreover, the hES cells can differentiate in vitro forming mature cells that represent all three germ lineages.[1]

These properties of hES cells make them a strong candidate for source material to derive specific cell types for cellular-based therapies for the treatment of human degenerative disorders. To enable development of such cellular-based therapeutics from the hES cells, it is important to show that the hES are stable in culture, can be grown in vitro under conditions suitable for scale-up, and can be differentiated and manipulated in vitro to specific cell types. In this report, we review results

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indicating that the hES cells can be stably propagated in culture for more than 50 passages. The cells can now be grown without the use of mouse embryonic feeder layers yet still maintain their ability to differentiate in immunocompromised animals and in vitro into cells that represent all three germ lineages.[3] Last, the hES cell can be genetically modified both transiently and long term, providing methods to engineer the cells for therapeutic applications.

MATERIALS AND METHODS

Maintenance of Undifferentiated Cells

The H1, H7, H9, and H14 human ES cells were maintained on primary mouse embryonic fibroblasts (MEFs) in serum-free media.[2] In brief, hES cells were seeded as small clusters on irradiated mouse embryonic fibroblasts seeded at about 40,000 cells/cm^2. hES cultures were maintained in an embryonic stem cell medium (ESM) composed of 80% knock-out Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD) and 20% serum replacement (Gibco) supplemented with 1% nonessential amino acids, 1 mM of glutamine, 0.1 mM of β-mercaptoethanol, and 4 ng/mL of human basic fibroblast growth factor (hbFGF) (Gibco). Cells were expanded by serial passage of the ES colonies. ES colonies were incubated in 200 μ/mL of collagenase IV for 15–20 minutes at 37°C. The cultures were then gently scraped, dissociated, and replated as small clusters onto fresh feeder layers.

For culture in the absence of feeders, the hES cells were collected after collagenase treatment as described earlier and seeded at 90,000 to 170,000 cell/cm^2 into Matrigel- or laminin (Gibco)–coated plates in conditioned medium. The conditioned medium was prepared from mouse embryonic fibroblasts used before passage 5 or from NHG190 cells, a G418-resistant, green fluorescent protein (GFP)–positive mouse embryonic cell line transfected with human telomerase. MEFs were maintained in 90% DMEM (Gibco), 10% fetal bovine serum (FBS) (Hyclone, Logan,UT), and 2 mM of l-glutamine. The NHG190 cells were maintained in the MEF medium supplemented with an additional 10% FBS. The cells were harvested, irradiated with 40 Gy, and seeded at 55,000 and 38,000 cells/cm^2 for the MEF and NHG190 cells, respectively. After at least 4 hours, the medium was exchanged with ESM (0.5 mL/cm^2). The conditioned medium was collected daily, supplemented with an additional 4 ng/mL of hbFGF, and used immediately for feeding hES cultures. The conditioned medium can also be frozen and thawed. Cells for generating conditioned medium were refed with ESM daily and used for 7–10 days.

For growth curve determination, replicate wells of known numbers of hES cells were established. At various time points after seeding, three wells were harvested and counted by use of a hemacytometer. Parallel plates of unseeded mouse embryonic feeders were counted at each timepoint to enumerate the number of feeder cells in the cultures.

Karyotype Analysis

Karyotype analysis was performed by GTG-banding analysis of 20 cells per population at Children's Hospital, Oakland, CA.

Relative Quantitation by Polymerase Chain Reaction

RNA was prepared with the QiaGen RNeasy kit. Before reverse transcription, RNA samples were digested with DNase 1 to remove contaminating genomic DNA. Reverse transcription reactions were performed with 500 ng of total RNA by use of random hexamers as primers and Superscript II reverse transcriptase (Bethesda Research Labs). For quantification of individual gene products by radioactivity, QuantumRNA Alternate18S Internal Standard primers (Ambion) were employed according to the manufacturer's instructions. Briefly, the linear range of amplification of a particular primer pair was determined, and then those primers were used in coamplification reactions with the appropriate mixture of alternate 18S primers: competimers to yield polymerase chain reaction (PCR) products with coinciding linear ranges. AmpliTaq (Roche) was preincubated with the TaqStart antibody (ProMega) according to manufacturer's instructions before initiation of PCR reactions. Radioactively labeled PCR products were resolved on 5% nondenaturing polyacrylamide gels, dried, exposed to phospho-image screens (Molecular Dynamics) for 1 hour, and scanned with a Molecular Dynamics Storm 860. Band intensities were quantified and normalized to the 18S signal by use of ImageQuant software (Table 1).

Microarray Analyses

All microarray analyses were performed under contract with Incyte Microarray services (Fremont, CA). PolyA$^+$ messenger RNA from undifferentiated ES cultures and embryoid body time course series was prepared as described[4] and sent to Incyte for probe preparation and hybridization to UNIGEM 1.0 microarrays. A complete description of the composition of the UNIGEM 1.0 array and methodologies can be viewed at *http://www.genomesystems.com/expression/index.html*. Signals less than 2.5-fold above background in both Cy5 and Cy3 channels were excluded from analysis. A balance factor was applied to normalize the total signal in the Cy5 and Cy3 channels.[4]

TABLE 1

Gene	Primers	Alternate18: Competimers	PCR Profile
OCT-4	Sense 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3' Antisense 5'-CTGCAGTGTGGGTTTCGGGCA-3'	1:4	19 cycles (94° 30 seconds; 60° 30 seconds; 72° 30 seconds)
hTERT	Sense 5''-CGGAAGAGTGTCTGGAGCAA-3' Antisense 5'-GGATGAAGCGGAGTCTGGA-3'	1:12	34 cycles (94° 30 seconds; 60° 30 seconds; 72° 30 seconds)

Abbreviation: PCR, Polymerase chain reaction.

hES Differentiation

Differentiation was induced by the addition of differentiation agents to monolayer hES cultures or the formation of Ebs. Differentiation of monolayer cultures was performed by adding 1% dimethyl sulfoxide (DMSO) or 1 μM of all *trans*-retinoic acid to the media. Embryoid bodies were produced by harvesting confluent cultures of human ES cells and plating in nonadherent cell culture plates (Costar) in differentiation medium composed of 80% KO DMEM (Gibco) and 20% non–heat-inactivated FBS (Hyclone) supplemented with 1% nonessential amino acids, 1 mM of glutamine, and 0.1 mM of β-mercaptoethanol. Four-day embroid bodies were transferred into gelatin-coated plates and maintained for an additional 7 days. The cultures were then processed for immunohistochemical analysis.

Teratoma Analysis

For teratoma analysis, approximately 5×10^6 cells from each of the four hES cell lines were injected intramuscularly into severe combined immunodeficiency disease (SCID)/beige mice. Seventy-eight to 84 days after injection, the tumors were excised and processed for histologic analysis. Histologic analysis was performed by IDEXX Corp, West Sacramento, CA.

Flow Cytometry

Unmodified and transfected hES cells were harvested at confluence using 0.5mM of ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and resuspended at approximately 1×10^6 cells/test. Cells were washed in a solution containing PBS plus 2% FBS, 0.1% sodium azide, and 2 mM of EDTA. Staining was performed in the same buffer with the addition of the appropriate antibodies. The mAB-recognizing SSEA-4 (MC813, the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) was used at 1:15 to 1:5 dilutions. Isotype matched controls were obtained from Sigma (St. Louis, MO). Cells were incubated with antibodies in a final volume of 100 μL for 30 minutes at 4°C, washed and incubated with a goat anti-mouse immunoglobulin M antibody conjugated with phycoerythrin (Southern Biotechnology Associates). Samples were washed as before, resuspended in the same buffer plus 1 μg/mL of propidium iodide (Sigma), and analyzed on a FACScalibur Flow Cytometer (Becton Dickinson) using CellQuest software. A total of 10,000 PI-negative events were collected per analysis.

Immunocytochemistry

Cultures were fixed for 10–20 minutes with 4% paraformaldehyde in PBS, washed three times with PBS, permeabilized for 2 minutes in 100% ethyl alcohol, and washed with 0.1 M of PBS. Cultures were then incubated in a blocking solution of 0.1 M of PBS with 5% normal goat serum (NGS, DAKO) and 0.1% Triton X-100 (Sigma) for at least 1 hour at room temperature. Cultures were then incubated in primary antibodies diluted in 0.1M of PBS with 1% NGS and 0.1% Triton X-100 for at least 2 hours at room temperature. The cultures were washed in PBS before 30-minute incubation with secondary antibodies in the same buffer.

The primary antibodies used recognized β-tubulin III, 1:1000 (Sigma), and alpha-fetoprotein, 1:500 (Sigma). The secondary antibodies used were goat anti-mouse FITC, 1:128 (Sigma) and goat anti-rabbit TRITC, 1:100 (Cappel).

Immunostaining for cardiac troponin I(cTnI) required fixation in methanol/acetone (3:1) at -20°C for 20 minutes. The cells were then washed two times with PBS, blocked with 10% NGS in PBS at 4°C overnight, followed by incubation at room temperature for 1 hour with a monoclonal antibody against cTnI (Spectral Diagnostic) diluted 1:300 in the primary antibody diluting buffer (Biomeda Corp). After washing, the cells were exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:100 in PBS containing 1% NGS at room temperature for 30 minutes.

The cells were then washed again and viewed by microscopy.

Genetic Modification of hES Cells

For transient transfection, the hES cells were seeded in six well plates in the absence or presence of feeder layers and genetically modified using FuGene 6 (Roche) reagent according to the manufacturer's protocol. For these experiments, the pEGFP-C1 plasmid (Clontech), containing the gene encoding the Green fluorescent protein (GFP) driven by the cytomegalovirus immediate early gene promoter, was used to genetically modify the cells. Four hours after the addition of the Fugene-DNA complex, ESM was added to each well. Twenty-four hours after transfection, the cells were harvested and analyzed by either fluorescence microscopy or flow cytometry to assess successful gene transfer and expression.

For stable transduction, the hES cells were plated in 24 well culture dishes on neomycin phosphotransferase–resistant NHG190 feeders and transduced using a VSV-G pseudotyped retrovirus vector, GRN354, which contains both the *GFP* gene and the neomycin phosphotransferase (neo^r) gene in a murine stem cell virus (MSCV)- based retroviral backbone (Clontech). In this vector, the GFP gene is driven by the MSCV long terminal repeat, and the neo^r gene is expressed by the murine pgk promoter. Four days after transduction, ESM medium containing 200 μg/mL of G418 (Geneticin, Gibco) was added to the cells. G418-resistant cells (H7NG) were selected and maintained in the same growth conditions.

RESULTS

hES Cultures

The hES cell lines were derived and propagated by culture on irradiated primary mouse embryonic feeders. These cells have been propagated in culture for more than 50 passages, yet maintain a normal karyotype. Table 2 details the number of passages and the karyotype of the different hES cell lines under feeder or feeder-free culture. In designated experiments, the hES cells were transferred to feeder-free cultures, where medium conditioned by mouse embryonic cells was used to support hES proliferation. The approximate number of population doublings was calculated using the passage number, the frequency of passage (once per week), and an average doubling time of 35 hours. A typical growth curve for the proliferation of the hES cells is shown in

TABLE 2 Duration of hES Culture and Karyotype Analysis

Cell Line	Growth Substrate	Passage Number	Approximate Population Doublings	Karyotype
H1	Feeders	32	154	Normal
H7	Feeders	29	139	Normal
H9	Feeders	32	154	Normal
H9	Feeders	40	192	Normal
H1	Feeders to P17	51	240	Normal
	Feeder free to P20	20	96	Normal
H1	Feeders to P17			
	Feeder free to P46	46	221	Normal
H7	Feeders to P19			
	Feeder free to P 32	32	154	Normal
H7	Feeders to P15			
	Feeder free to P22	22	106	Normal
H9	Feeders to P30			
	Feeder free to P34	34	163	Normal
H9	Feeders to P14			
	Feeder free to P18	18	86	Normal
H9	Feeders to P14			
	Feeder free to P22	22	106	Normal
H9	Feeders to P14			
	Feeder free to P43	43	206	Normal
H14	Feeders to P16			
	Feeder free to P19	19	91	Normal

Abbreviation: hES, human embryonic stem.

Figure 1. Similar results were obtained in seven such experiments testing various hES lines in which the doubling time during log phase growth ranged from 28–50 hours.

Gene Expression in hES Cells

To more thoroughly characterize the hES cells, specific gene expression was monitored as a function of differentiation. Both *OCT-4* and *hTERT* expression were detected in all undifferentiated human ES lines examined. To examine the effects of differentiation induction on *OCT-4* and *hTERT* expression, H7 and H9 hES cells were exposed to retinoic acid (RA) or DMSO. After 7 days of drug treatment, the cellular morphology in the cultures changed, with the gradual disappearance of undifferentiated cells. After 7 days of exposure to either drug, both *OCT-4* and *hTERT* expression were reduced (Fig. 2). More dramatic decreases in *OCT-4* and *hTERT* expression were observed with DMSO treatment that were consistent with its more rapid induction of differentiation of the culture.

To more thoroughly characterize baseline hES gene expression, genes preferentially expressed in either the undifferentiated or partially differentiated cells were identified and categorized by microarray analysis. For these experiments, differential gene expression analysis was performed by contrasting messenger RNAs from undifferentiated H9 cultures with that from 2d, 4d, or 8d embryoid bodies. In these studies, embryoid bodies were either maintained in suspension in differentiation medium for 8 days or kept in differentiation medium for 4 days, followed by 4 days of treatment with 0.5 μM of retinoic acid. The arrays used in these experiments include approximately 10,000 complementary DNAs selected to represent a large portion of characterized human genes. As seen in Figure 3, the differentiation of hES cells involves the activation and repression of many genes, including expressed sequence tags (ESTs) that lack significant annotation. The addition of RA to the embryoid bodies had relatively minor effects on the gene expression pattern (compare 4d-/4d+ with 8d).

Genes showing reduced expression during differentiation include metallothioneins, growth factors (*FGF 9*), secreted cysteine-rich proteins (*osteopontin, AGF-BP5, Cyr61, connective tissue growth factor*), and the *selenium donor protein selD*. In general, the most significant decreases in expression on differentiation occur after 4 days of suspension culture. The expression of *uridine diphosphate–glucose phosphorylase* and *phosphoglucomutase*, two genes involved in alpha-d-Glucose phosphate catabolism, are dramatically reduced, suggesting a potential alteration in glucose metabolism.

The microarrays used for these experiments did not contain the human telomerase gene, although human telomerase reverse transcriptase PCR and activity measurements indicate that telomerase expression decreases significantly on differentiation. However, microarray analysis did show that differentiation was associated with a marked decrease in the expression of *TRF1*, a principal telomere-binding factor, whose expression has been correlated with telomere shortening.[5] Thus, the expression of both positive (*hTERT*) and negative (*TRF1*) regulators of telomere length diminishes during hES cell differentiation.

Several genes associated with visceral endoderm and early hepatic differentiation (*AFP, apoplipoprotein A-II, apoplipoprotein AI regulatory protein-1, α1-antitrypsin,* and the *α, β,* and *γ gamma chains of fibrinogen*) were induced within 2 days of differentiation; this induction is not substantially affected by RA. Of note, no induction of cellular retinoic acid binding proteins 1 and 2 (CRABP1, 2) was observed in RA-treated cultures, consistent with a proposed negative feedback loop in which retinoids inhibit CRAB I gene promoter activity.[6] Other genes induced by differentiation include *pleiotropin* and *midkine*, secreted cytokines with proposed roles as mitogens for neuronal, hepatic, and endothelial cells.[7,8] As such, these factors may play similar roles in ES cell differentiation. The induction of DNA binding proteins (homeobox b5 protein, *meis1*) likely reflects the central role of transcriptional regulators in differentiation. The

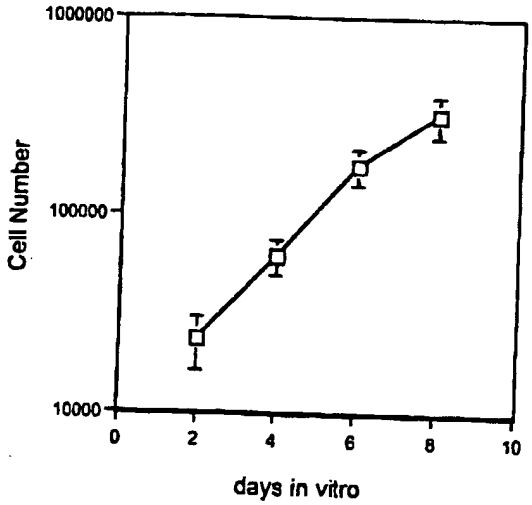

FIGURE 1 Growth of human embryonic stem (hES) cells. H9 hES cells were plated in multiple wells in 24-well culture dishes. Cells were harvested at days 2, 4, 6, and 8 of culture and enumerated by use of a hemocytometer. Parallel plates of cells containing only feeders were also counted at each time point to establish the contribution of feeders to the overall cell number. The number of feeders was subtracted from the overall cell number at each timepoint. The irradiated feeders did not proliferate during the culture period. The doubling time was calculated from the slope of the growth curve.

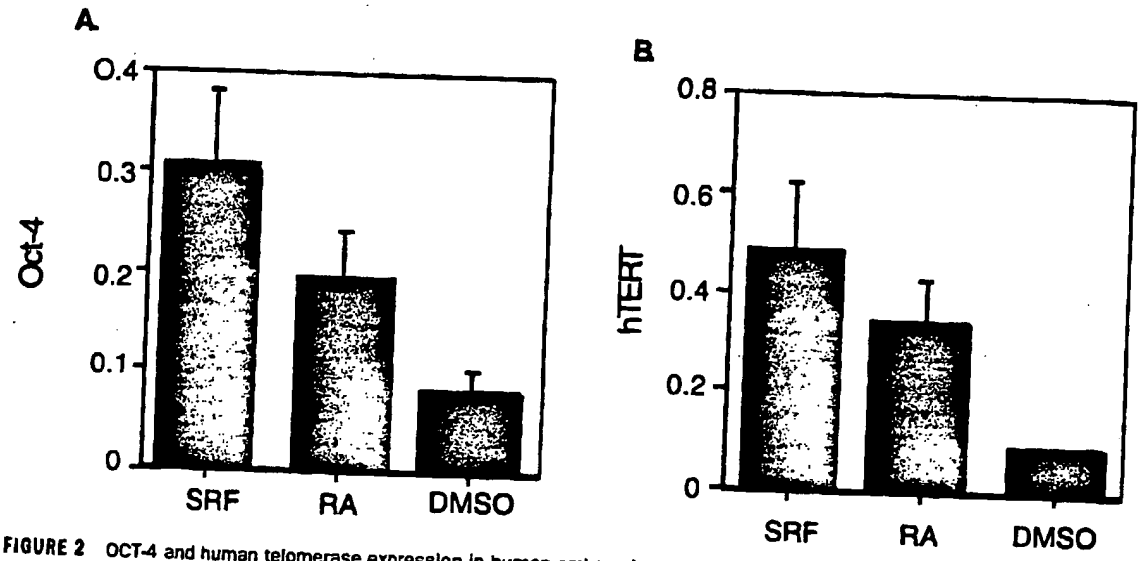

FIGURE 2 OCT-4 and human telomerase expression in human embryonic stem (hES) cells. H9 cells were grown in ESM, medium containing 0.5 μM of RA (all-*trans*-retinoic acid [Sigma], dissolved in ethanol), or 0.5% of dimethyl sulfoxide (ATTC) for 7 days. RNA was isolated, and quantitative polymerase chain reaction was used to determine relative changes in the expression of *OCT-4* (*A*) and *hTERT* (*B*).

expression of the gp130 IL6 receptor, which is essential for transduction of leukemia inhibitory factor (LIF) signals,[9] is low in hES cultures and is induced on differentiation.

Feeder-Free Culture

Despite its proliferative effects on mouse embryonic stem cell cultures, LIF does not stimulate the in vitro propagation of the undifferentiated hES cells (unpublished data, M. Carpenter, 1999).[1,10] As a result, mouse embryonic feeder layers have been required to maintain the proliferation of the undifferentiated hES cells. To improve the ease, reproducibility, and scalability of hES culture, methods were developed to culture the cells in the absence of feeders. For feeder-free culture, the hES cell lines were plated on Matrigel or laminin in the presence of medium conditioned by primary mouse embryonic cells or a telomerase-immortalized mouse embryonic cell line. Four hES cell lines, H1, H7, H9, and H14, have been propagated successfully on Matrigel in the presence of the conditioned medium. The cells maintained a doubling time of 31–35 hours, similar to that of the hES cells cultured in the presence of the mouse embryonic cell feeders. The hES cultures have been maintained for more than 30 passages in the absence of the feeder layers and continue to maintain a normal morphology and karyotype, as monitored by GTG-banding (Table 2). The hES cells cultured feeder free continue to express SSEA-4, Tra-1-60, Tra-1-81, OCT-4, alkaline phosphatase, and telomerase. Finally, the feeder-free hES cells were successfully cryopreserved in ESM medium supplemented with 10% DMSO and 30% KNOCKOUT SR using a controlled rate freezer.

Differentiation of hES Cells

The hES cells propagated in mouse embryonic cell–conditioned medium maintain their pluripotent potential and differentiate into cells of all three embryonic germ layers on appropriate stimulation. All of the hES cell lines cultured using conditioned medium and extracellular matrix factors formed embryoid bodies in vitro. For further differentiation, the embroid bodies maintained in suspension for 4 days were plated on gelatin-coated plates and cultured for an additional 7 days. After differentiation, the cells were fixed and stained with monoclonal antibodies to detect lineage-specific cells. Figure 4 shows that H1 hES cells maintained four passages without feeders using conditioned medium and were capable of differentiating into tissue representing all three germ line lineages. Ectoderm derivatives were observed by the presence of cells with long, distinct processes positive for the β-tubulin III marker (Fig. 4A). Likewise, differentiation of mesoderm tissue was detected by the presence of numerous beating structures in the culture. These beating cells stained positively for cardiac troponin I, indicative of cardiomyocytes in the culture (Fig. 4B). Finally, endoderm differentiation was observed as large distinct polygonal cells, which expressed alpha-fetoprotein (Fig. 4C). Similar results were observed on differentiation of the H7 and H9 cell lines.

To determine whether hES cells grown for extended periods without feeder cells would differentiate in vivo,

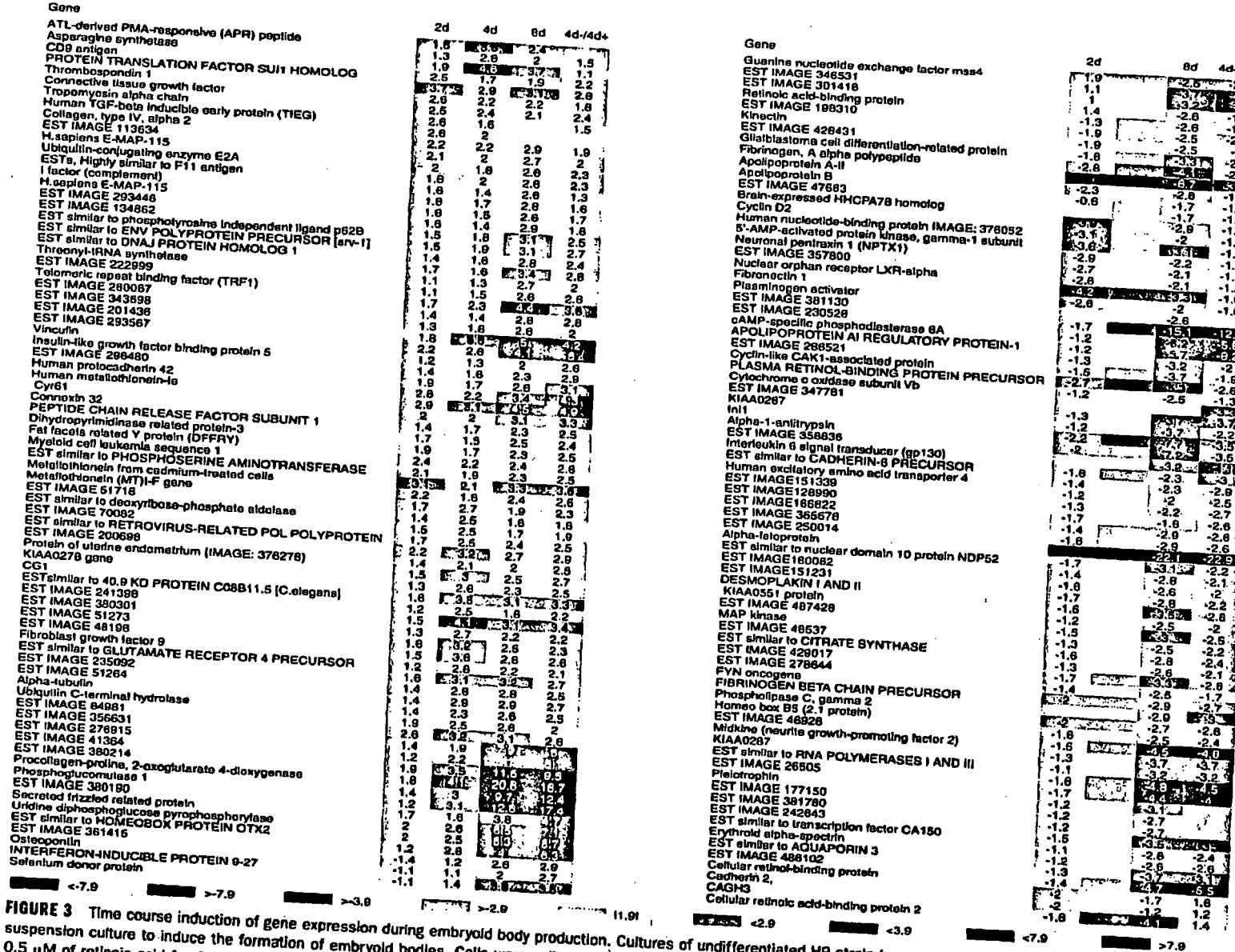

FIGURE 3 Time course induction of gene expression during embryoid body production. Cultures of undifferentiated H9 strain human embryonic stem (hES) cells were placed in suspension culture to induce the formation of embryoid bodies. Cells were collected at the indicated time points. After 4 days in suspension, cultures were either treated with 0.5 μM of retinoic acid for 4 additional days (4-/4+) or left untreated for 4 additional days (8d). Genes whose expression was increased during embryoid body production are red-shifted, and genes whose expression was reduced are green-shifted (*see color bar, lower*).

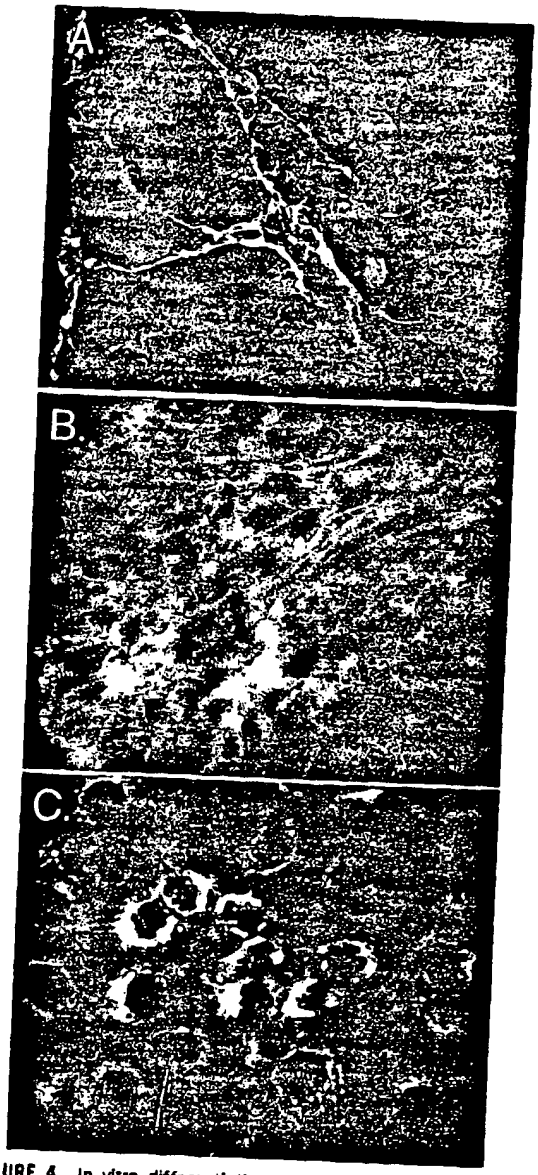

FIGURE 4 In vitro differentiation of human embryonic stem (hES) cells. H1 p50 hES cells were differentiated and analyzed by immunohistochemistry. A, β-Tubulin III–positive neurons. B, Cardiac troponin I–positive cardiomyocytes. C, Alpha-fetoprotein–positive endoderm.

cells from each of the hES lines were administered by intramuscular injection to severe combined immunodeficiency disease/beige mice for teratoma formation and analysis. These cells had been grown for a total of 24–29 passages, the last three to seven of which were in the absence of feeders. Each of the cell lines formed teratomas, which were harvested and analyzed 78–84 days after inoculation. Histologic analysis of the teratomas was completed, and representative photographs are presented in Figure 5. Each of the teratomas was composed of complex structures resembling immature tissues of the major organ systems of the adult (Figs. 5A and B). Representative tissues of the three germ lineages were observed in teratomas from each of the hES cell lines. Neuroepithelial tissue was observed in all of the teratomas, a finding that is indicative of ectoderm differentiation (Figs. 5C and D). Moreover, mesoderm differentiation was also observed as dental, cartilage, bone (Figs. 5E and F), and muscle tissue (data not shown). Finally, derivatives of the endoderm were also present in the teratomas with glomeruli, liver, and glandular tissue observed in the teratoma sections (Figs. 5G and H). The types of cells and structures were similar to those observed from hES cells maintained on feeders. Therefore, consistent with the in vitro results, the hES cells maintained in the absence of feeder layers remained pluripotent, forming tissues of all three germ layers.

Genetic Modification of hES Cells

The hES cells can be genetically modified both transiently and stably in vitro (Fig. 6). H9 cells genetically modified by use of a plasmid encoding the GFP protein and a lipofection reagent fluoresce green, as observed by fluorescence microscopy 24 hours after transfection (Figs. 6A and B). Undifferentiated hES cell transfection efficiencies were determined to be 30%–80%, as measured by flow cytometry of SSEA-4–expressing cells. Expression was transient: green fluorescence was diminished by 120 hours after transfection. In similar studies, the hES cells were also efficiently transduced by adenovirus vectors, with expression diminishing within days as the cells continued to proliferate (data not shown). Finally, hES cell lines were stably transduced by use of VSV-G–pseudotyped, MSCV-based retroviral vectors.[11] Transduced, drug-resistant cells were successfully selected and cultured in vitro. Figure 6D shows results from a drug-resistant, undifferentiated H7 cell line that was created by transduction with the neomycin phosphotransferase and *gfp* genes. The stably transfected cell line continued to express SSEA-4, and 50%–65% of the cells expressed the gfp protein (Fig. 6D). Control cells not transduced with the *gfp* gene did not fluoresce green (Fig. 6C). The stably transfected cells continued to express gfp after approximately 3 months and maintained their ability to differentiate into all three germ lineages. On differentiation, many of the cells express higher levels of gfp, presumably reflecting differential activation of the retroviral LTR in multiple cell types.

DISCUSSION

To realize the full potential of human ES cells, an understanding of the properties of the undifferentiated stem

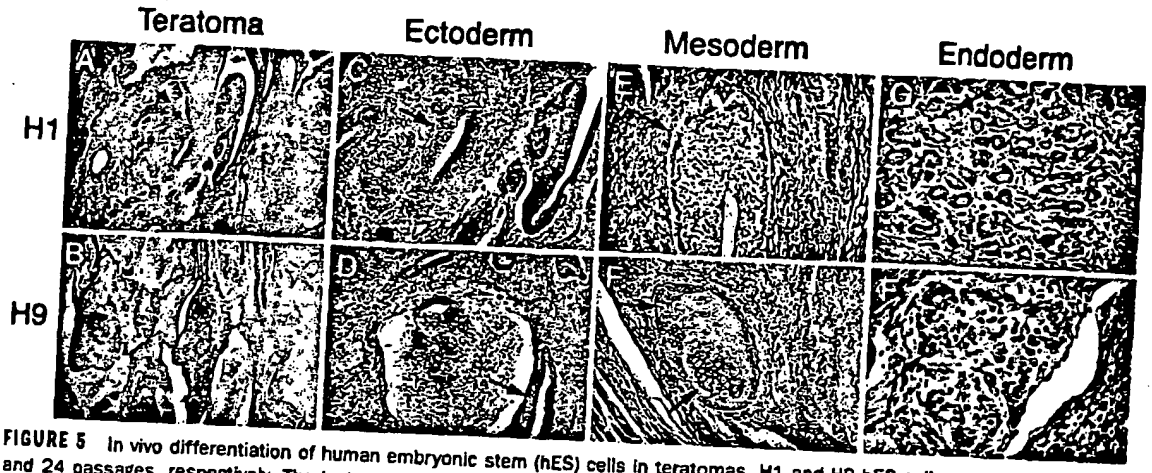

FIGURE 5 In vivo differentiation of human embryonic stem (hES) cells in teratomas. H1 and H9 hES cells were cultured for 26 and 24 passages, respectively. The last passages were performed in the absence of feeder layers. Teratomas were formed by injection of the hES cells into SCID/beige mice. Histologic analysis was performed. The top and bottom panels are photographs of structures from the H1 and H9 hES cell line, respectively. Panels A and B represent low-magnification (40×) photographs of the teratomas showing the presence of multiple tissue types of complex organization. Panels C and D show characteristic neuroepithelial ectodermal tissues (*arrows*) found abundantly in the teratomas. Panels E and F are photographs of characteristic mesodermal tissue with evidence of dental (panel E, *arrow*), cartilage, and bone (panel F, *arrows*). Panels G and H show endoderm derivatives with the presence of glandular (panel G, *arrow*) and liver hepatocytes (panel H, *arrow*). Panels C to H are at 100×.

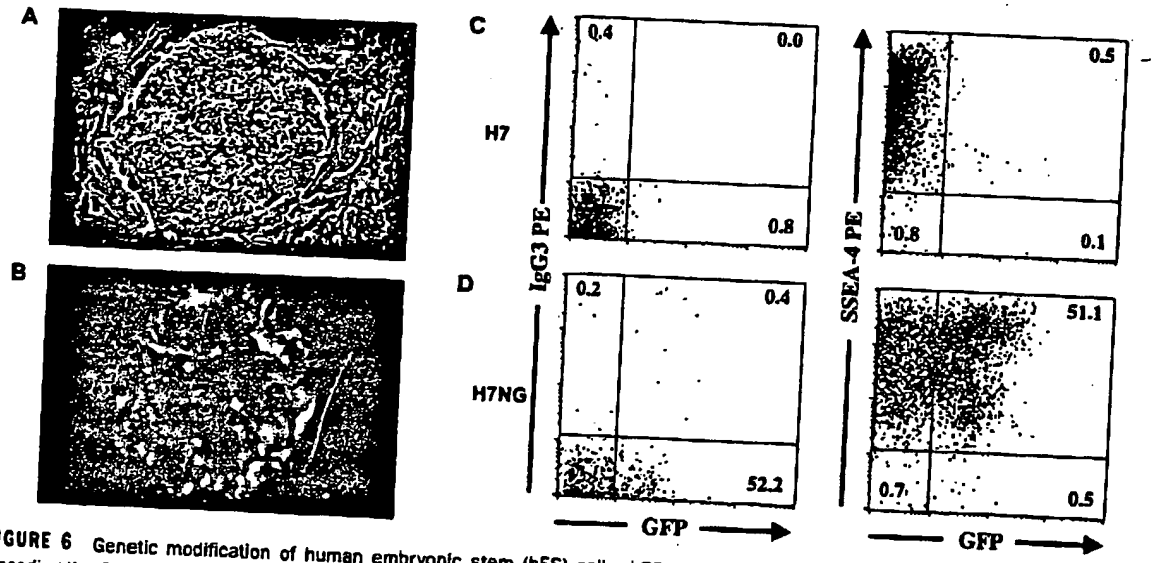

FIGURE 6 Genetic modification of human embryonic stem (hES) cells. hES cells were transiently transfected with a plasmid encoding the *GFP* gene. Twenty-four hours after transfection, the cells were observed by use of fluorescent microscopy. A, Transfected hES colony in bright phase. B, Transfected hES colony fluorescing green during fluorescent microscopy. C and D, Stable transduction of H7 cells was performed. The control H7 (C) and transduced H7NG cells were stained with an antibody to SSEA-4. SSEA-4 (red) and gfp (green) fluorescence was monitored by two-color flow cytometry.

cells as well as the development of scaleable methods to reproducibly propagate and differentiate these pluripotent cells is required. To this end, we have studied the proliferative capacity of these cells and determined the stability of the cells, as measured by karyotype analysis. The hES cells can be cultured in vitro for more than 250 population doublings and maintain a normal karyotype throughout extensive ex vivo culture. The hES cells continue expression of SSEA-4, Tra 1-60, Tra 1-81, and genes that have been implicated in the persistence of the totipotent state (*OCT-4*) or in replicative immortality (*hTERT*). We also demonstrate that

expression of both *OCT-4* and *hTERT* is repressed by agents that induce differentiation. The similar expression patterns of these distinct genes may imply a common pathway by which hES cells regulate the expression of genes that are characteristic of the undifferentiated state. The ability to measure quantitative changes in these two low-abundance genes facilitates sensitive screens for the detection of culture conditions, soluble factors, or transfected genes that inhibit or enhance differentiation.

DNA microarray technology enables the simultaneous evaluation of the expression patterns of tens of thousands of genes. In many cases, information on the function or tissue distribution of genes is extremely limited; identification of these ESTs as genes whose expression changes during hES cell differentiation may reflect their roles in early human embryogenesis. Of particular interest are the genes that show diminished expression in the early stages of differentiation. One or more of these genes may be responsible for maintaining the developmental plasticity of undifferentiated stem cells. The identification of such genes may provide information that could be useful for inducing the reprogramming of somatic cells, allowing the derivation of immunologically matched tissues for transplantation. In contrast, some genes showing increased expression during hES cell differentiation may play roles in the development of specific lineages. Determination of the processes by which many lineages are formed has been difficult due to the lack of availability of early-stage tissue or precursor cells. The ability to recapitulate early-stage differentiation in vitro with hES cells will permit characterization of these developmental steps. The combination of culture conditions that induce the efficient differentiation of hES cells to specific cell types and the application of DNA microarray technology will allow the elaboration of the series of genetic events by which an unspecified cell becomes committed to a restricted cell fate and the process by which that specific mature cell type is achieved.

In vitro differentiation of ES cells provides a potential source of cells and tissues for in vitro drug screening, toxicology testing, and therapeutic transplantation. To reliably provide the cells for these applications, precise, controlled, scalable processes must be developed to cultivate and differentiate the hES cells. To this end, we have described a process for propagating the hES cells without the use of feeder cells. In this process, the feeder cells are replaced by extracellular matrix factors along with conditioned medium. hES cells have now been cultivated for months without the use of feeders. These cells maintain gene expression patterns similar to that of hES cells cultivated on feeders and retain their ability to differentiate into cells of all major germ lineages.

Elimination of feeders substantially improves the scalability and ease of hES cell culture. First, the use of conditioned medium eliminates feeder cell contamination of the hES cells, improving the homogeneity of the final product for all applications. In addition, the conditioned medium can be produced in large lot sizes, can be quality controlled, and can be stored long term for relatively uniform production of cells. The use of telomerase-immortalized cells for the production of the medium is particularly well suited for the large-scale production of conditioned medium. Finally, the successful use of conditioned medium allows the characterization of factors that may be present or specifically depleted in the conditioned medium to more truly define the factors necessary for undifferentiated cell maintenance.

Finally, processes have been described for both transient and stable genetic modifications of the hES cells. Genetic modification of hES cells will be critical for many applications. Genetic modification of hES cells could lead to the creation of cell lines, which could be used to screen differentiation paradigms to improve the efficiency of producing mature cell populations. Moreover, such cell lines could be used to screen drug candidates and test for their toxicity in vitro. Genetic modification could lead to both the positive and the negative selection of specific cell derivatives of hES. In addition, telomerase gene transfer into differentiated components of hES cells could lead to the immortalization of precursors or other mature subsets of hES cells. Gene transfer into hES cells will be valuable for functional genomics applications. Finally, gene transfer into hES cells and their derivatives could prove valuable to reduce the immunologic rejection of hES-derived transplants in recipients. Key areas for future research include the development of expression systems, which will permit regulatable and/or permanent expression in targeted cell types.

The improvements in the culture and manipulation of hES cells described here are directly suitable for bioreactor scale-up. These technological developments are important steps to realizing the potential of hES cells for therapeutic applications.

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